brief communication

# Mode of action of iberiotoxin, a potent blocker of the large conductance $Ca^{2+}$-activated $K^+$ channel

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ABSTRACT Iberiotoxin, a toxin purified from the scorpion *Buthus tamulus* is a 37 amino acid peptide having 68% homology with charybdotoxin. Charibdotoxin blocks large conductance $Ca^{2+}$-activated $K^+$ channels at namolar concentrations from the external side only (Miller, C., E. Moczydlowski, R. Latorre, and M. Phillips. 1985. *Nature (Lond.).* 313:316–318). Like charybdotoxin, iberiotoxin is only able to block the skeletal muscle membrane $Ca^{2+}$-activated $K^+$ channel incorporated into neutral planar bilayers when applied to the external side. In the presence of iberiotoxin, channel activity is interrupted by quiescent periods that can last for several minutes. From single-channel records it was possible to determine that iberiotoxin binds to $Ca^{2+}$-activate $K^+$ channel in a bimolecular reaction. When the solution bathing the membrane are 300 mM $K^+$ internal and 300 mM $Na^+$ external the toxin second order association rate constant is $3.3 \times 10^6\ s^{-1}\ M^{-1}$ and the first order dissociation rate constant is $3.8 \times 10^{-3}\ s^{-1}$, yielding an apparent equilibrium dissociation constant of 1.16 nM. This constant is 10-fold lower than that of charibdotoxin, and the values for the rate constants showed above indicate that this is mainly due to the very low dissociation rate constant; mean blocked time ~ 5 min. The fact that tetraethylammonium competitively inhibits the iberiotoxin binding to the channel is a strong suggestion that this toxin binds to the channel external vestibule. Increasing the external $K^+$ concentration makes the association rate constant to decrease with no effect on the dissociation reaction indicating that the surface charges located in the external channel vestibule play an important role in modulating toxin binding.

## INTRODUCTION

Large conductance $Ca^{2+}$-activated $K^+$ [K(Ca)] channels belong to a class of $Ca^{2+}$-activated channels characterized by their sensitivity to externally added charybdotoxin (ChTX; Miller et al., 1985; for reviews see Latorre et al., 1989; Garcia et al., 1991). ChTX, originally found as a minor component of the scorpion *Leiurus quinquestratus* var *hebraeus,* is able to block, at nanomolar concentrations, by binding to a receptor located in the external mouth of K(Ca) channels (Anderson et al., 1988; Miller, 1988). ChTX is a 37 amino acid peptide containing several positively charged residues given the peptide a net charge of +5 and having 6 cysteine residues (Fig. 1; for reviews see Moczydlowski et al., 1988; Garcia et al., 1991). The three-dimensional structure of ChTX has been recently solved by proton NMR (Lambert et al., 1990; Bontems et al., 1991). ChTX consists of a $\beta$-sheet linked to an extended strand by a disulfide bond and to an $\alpha$-helix by two of such bonds.

Despite the fact that ChTX is a high-affinity blocker of K(Ca) channels, its specificity is poor. ChTX also blocks voltage-dependent type $n$ and $n'$ $K^+$ channels of T lymphocytes with a $K_d$ ~ 1 nM (Sands et al., 1989), and some small conductance $Ca^{2+}$-activated $K^+$ channels (Hermann and Erxleben, 1987). The lack of selectivity of ChTX prompted the search of new and more specific toxins for K(Ca) channels. In particular, Galvez et al. (1990) found that the venom of the scorpion *Buthus tamulus* was able to modulate ChTX binding to smooth muscle sarcolemmal membranes. Fractionation of this venom and purification of the active component gave as a result a peptide displaying 68% sequence identity with ChTX, but possessing four more negatively charged (Asp) and one less positively charged residue (Fig. 1).

In the present work we have studied the mode of action of this peptide christened Iberiotoxin (IbTX, Galvez et al., 1990). We show that like ChTX, IbTX is able to block K(Ca) channels by binding to their external mouth. Blockade is characterized by the appearance of long-lived nonconducting states separating bursts of channel activity. At difference of ChTX where the quiescent periods induced by the toxin last seconds, the long-lived closings induced by IbTX can last several minutes. Binding of IbTX to the external mouth of the channel is tighter than that of ChTX, and this is a consequence of a much smaller dissociation rate constant. Similar results have been reported by Giangiacomo et al. (1991) using a K(Ca) channel from bovine aortic smooth muscle.

Part of this work has been presented in abstract form elsewhere (Candia et al., 1991).

## METHODS

### Biochemical

Membrane vesicles from rat skeletal muscle were prepared according to a modification of the procedure of Rosemblatt et al. (1981) described in detail by Moczydlowski and Latorre (1983). Membranes were stored in 30 $\mu$l aliquots in a solution containing 20 mM Tris-maleate, 300 mM sucrose, pH 7.0 at −80°C. The lipid was bovine brain phosphatidylethanolamine (PE) from Avanti Polar Lipids, Inc. (Birgmingham, AL) and were used as a solution containing 10 mg lipid per ml of decane. IbTX was purified from the lyophilized venom of *Buthus tamulus* (Sigma Chemical Co., St. Louis, MO) according to the procedure described by Galvez et al. (1990). IbTX was stored in lyophilized form and dissolved in the appropriate buffer before use.

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FIGURE 1 Primary structure of charybdotoxin (ChTX) and iberiotoxin (IbTX). The portions of the sequence enclosed in rectangles are regions of identity between the two toxins.

## Bilayer formation and channel incorporation

Planar bilayers were formed from the PE solution in a ~300-$\mu$m diameter hole made in a Teflon partition (18 $\mu$m in thickness) separating two aqueous compartments. Bilayers electrical capacitance ranged from 250 to 300 pF. K(Ca) channels were inserted into the planar bilayer by adding the membrane vesicles to one of the aqueous compartments that contained 300 mM KCl and 20 mM MOPS-KOH, pH 7 whereas the other compartment contained only the buffer. Channel insertion was detected as current jump of ~10 pA. After channel incorporation, and to avoid further vesicle fusion, the concentration gradient was completely dissipated by adding the appropriate amount of 3 M KCl or 3 M NaCl to the compartment containing only the buffer. In some cases, where the effect of ionic strength on the toxin effect was studied, the diluted membrane side contained 5 mM MOPS-KOH, pH 7. Most often, channels were incorporated into the bilayer leaving their cytoplasmic face (where the $Ca^{2+}$ activation sites are located) towards the side to which the vesicles were added. However, in those few exceptions in which the orientation of the channel was not the usual, this was easily determined by the polarity of their voltage dependence [K(Ca) channels open with depolarizing voltages or by their sensitivity to $Ca^{2+}$. In all experiments reported here the physiological voltage convention was used. i.e., the electrical ground is defined on the external side of the channel.

The voltage clamp circuit has been described in detail (Alvarez, 1986). The current across the bilayer was measured with a low-noise current-to-voltage converter, filtered at 1 kHz with an eight-pole Bessel low-pass active filter, amplified and recorded continuously on video tape.

## Data analysis

For analysis of "fast" channel kinetics in control experiments or during a burst in experiments done in the presence of IbTX, records were acquire at 200 $\mu$s/point and open and closed events were identified using a discriminator located at 50% of the open channel current. The durations of the open state and the closed state were corrected taking into account the dead time of the system as described by Colqhoum and Sigworth (1983). Dwell-time histograms were constructed using the method described by Sigworth and Sine (1987). The dwell time histograms were logarithmically binned and fitted by a sum of exponential probability functions using maximum likelihood. In this type of representation, peaks correspond to the time constant of the distribution considered.

Blocked and unblocked (burst) dwell times were determined directly from the single-channel current records. Long-lived blocked events were unambiguously recognized as quiescent periods lasting for several seconds, because in the most unfavorable cases (small voltages and contaminant $Ca^{2+}$ concentrations) channel closing events were short-lived (≤300 ms; see below). A burst or active period is defined as a period of channel activity flanked by two blocked periods. The association and dissociation rates of IbTX were obtained either from the mean blocked or mean active (burst) times obtained from the cumulative distribution of dwell times. The exponential fits to the experimental dwell time distributions were done using a nonlinear curve fitting program. The mean times were also obtained from the population mean defined as: mean = $\Sigma$ [dwell times in blocked (active) state]/number of blocked (active) events. Both methods gave essentially the same results as expected from the kinetic model described below (e.g., Vergara and Latorre, 1983, Anderson et al., 1988). Bilayers containing a single K(Ca) channel are typically stable for one hour and the probability of keeping a single-channel membrane for several hours, as required here due to the extreme slowness of the blocking process, is low. Due to this experimental limitation, we were forced to base the analysis on only 20–80 events per single-channel membrane.

## RESULTS

Fig. 2 *A* shows the channel activity during a control period of several minutes. In the absence of IbTX, the channel fluctuates rapidly between two conductance states, and this type of channel behavior remains unchanged during the entire recording period in this condition. As is well known (Moczydlowski and Latorre, 1983), K(Ca) channels show spontaneous shifts in the open probability ($P_o$). Due to this potential problem, only channels with a stable $P_o$ during the control period, as that shown in Fig. 2 *A*, are considered in the present study. The inhibition of K(Ca) channels by IbTX is illustrated in Fig. 2 *B*. The toxin induces the appearance of long periods of time in which the channel remains quiescent (~220 s; Table 1); these long-lived closed state separate bursts of channel activity.

Addition of IbTX to the internal side of the channel to final concentrations 10-fold larger than that employed in the experiment depicted in Fig. 2 *B*, did not cause the appearance of this very long-lived nonconducting state (data not shown). On the other hand, the effect of externally added IbTX can be reversed by extensive perfusion of the external compartment with a toxin-free buffer. For the sake of comparison, Fig. 2 *C* shows blocked intervals caused by charybdotoxin in a different K(Ca) channel, under similar experimental conditions. Blocked periods induced by ChTX are shorter (~20 s) than those caused by IbTX.

The gating kinetics inside the characteristics bursts found after addition of IbTX have the same kinetic components as those found during the control period (Fig. 3). In both cases, after filtering at 1 kHz, we found only one open state and three closed states. Open and closed distributions before and after addition of the toxin have essentially the same time constants. Notice that the longest closed state in the control period or inside a burst has mean time of 172 and 235 ms, respectively. This observation tells us that block events induced by IbTX can be easily distinguished from channel closures. Actually, the mean blocked time is about 1,000-fold longer that the slowest closed gating component (see below).

## IbTX binds to K(Ca) channels in a bimolecular reaction

The most simple and testable kinetic model for the mode of action of IbTX that comprises the bursts (active chan-

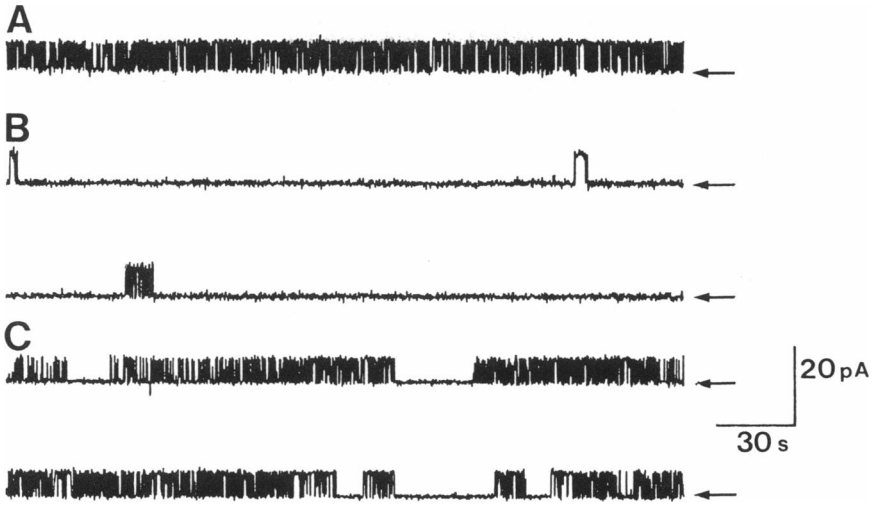


FIGURE 2 IbTX induces a long-lived closed state. (*A*) Control single-channel current record taken in the presence of 300 mM KCl, 67 $\mu$M $CaCl_2$, 5 mM MOPS-KOH, pH 7 in the internal side and 350 mM NaCl, 5 mM MOPS-KOH, pH 7 in the external side. $P_o = 0.70$. (*B*) After a control period of $\approx$10 min, IbTX was added to the external side to a final concentration of 18 nM. Holding potential: 0 mV Other experimental conditions as in *A*. A clear burst of channel activity lasting $\sim$15 s is seen in the second record. (*C*) Typical current record taken in the presence of 17 nM ChTX in the external side obtained in a different single-channel membrane. Internal side contained: 300 mM KCl, 80 $\mu$M $CaCl_2$, 20 mM MOPS-KOH, pH 7. External side contained: 300 mM NaCl, 20 mM MOPS-KOH pH7. $P_o$ of the control record was 0.6. Holding potential was 0 mV. Notice that blocked periods are much shorter than those induced by IbTX. The arrows indicate the closed state.

nel) and the long-lived closed periods induced by the toxin is given below.

$$\text{active channel} \underset{k_{-1}}{\overset{k_1[\text{IbTX}]}{\rightleftharpoons}} \text{blocked channel.} \qquad \text{(R1)}$$

According to reaction scheme R1, both the dwell times in the blocked state as well as the burst residence times must be distributed as single exponentials. This prediction is confirmed by the experimental results illustrated in Fig. 4, *A* and *B*, that show that the dwell-time distributions for the active and for the blocked channel are exponential. The model also predicts that the mean burst time, $\tau_A$, is given by

**TABLE 1 Characteristics of IbTX kinetics: effect of ionic strength**

| Ionic composition external side | $k_1$ | $k_{-1}$ | $K_d$ |
|---|---|---|---|
| *mM* | $s^{-1}\,M^{-1}$ | $s^{-1}$ | *nM* |
| 12.5 $K^+$ (2, 36) | $1.2 \times 10^7$ | $5.2 \times 10^{-3}$ | 0.47 |
| 310 $K^+$ (2, 22) | $1.1 \times 10^6$ | $4.0 \times 10^{-3}$ | 3.64 |
| 2.5 $K^+$ + 300 $Na^+$ (5, 100) | $3.3 \times 10^6$ | $3.8 \times 10^{-3}$ | 1.16 |
| 50 $K^+$ + 300 $Na^+$ (4, 66) | $1.8 \times 10^6$ | $4.1 \times 10^{-3}$ | 2.27 |
| 150 $K^+$ + 300 $Na^+$ (2, 42) | $1.6 \times 10^6$ | $3.3 \times 10^{-3}$ | 1.99 |

The ionic composition of the internal side was 300 mM, 10 mM MOPS-KOH, pH 7 in all cases. In those cases where only $K^+$ was present in the external side, data were obtained at 10 mV. When data was obtained in the presence of 300 mM external $Na^+$ the holding potential was 0 mV. The first number in the parenthesis refer to the number of different single-channel membranes; the second number is the total number of transitions.

$$\tau_A = 1/k_1[\text{IbTX}]. \qquad (1)$$

Notice that in scheme R1, toxin binding proceeds without making a distinction whether the channel is in the closed or the open state. Anderson et al. (1988) showed that ChTX binds about eightfold faster to the open channel than to the closed channel. If IbTX behaves as ChTX and can bind to both states, the general expression for the association rate constant when there is not interconversion between the states blocked-closed and blocked-open is: $k_1 = k_o P_o + k_c(1 - P_o)$, where $k_o$ and $k_c$ are the true second order association rate constants with the open and closed channel configurations respectively (Vergara and Latorre, 1983; Anderson et al., 1988). We have tried to avoid the potential problem of a possible dependence of the binding with $P_o$ by working always at $P_o$'s $\geq$ 0.7, i.e., we are mostly characterizing the IbTX binding to open state. On the other hand, the mean blocked time, $\tau_B$, is given by the inverse of the rate constant for leaving the blocked state. i.e.,

$$\tau_B = 1/k_{-1}. \qquad (2)$$

Fig. 5 shows that the predictions of reaction scheme R1 are corroborated experimentally. Fig. 5 *A* shows that the reciprocal mean active time is directly proportional to the IbTX concentration; and Fig. 5 *B* illustrates that $1/\tau_B$ remains essentially constant over the entire range of [IbTX] tested. The above set of results strongly suggest that IbTX binds to the K(Ca) channel according to the simple kinetic scheme R1 indicating that each long-lived closed period is a time span in which a single IbTX molecule is tightly bound to the K(Ca) channel. The rates

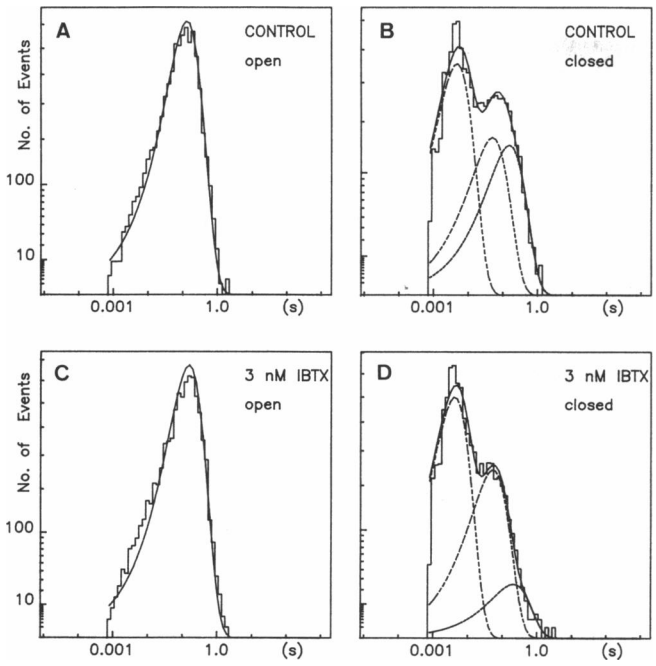


FIGURE 3 Channel gating kinetics during a burst in the presence of IbTX is similar to that found before addition of toxin. (*A*) Open time distribution from a channel current record taken in the absence of IbTX (control). Data are well fitted by a single component with a time constant of 150 ms. Total number of events considered in the analysis were 7499. (*B*) Closed time histogram from 7,500 events for the control period. Time constants for the different distribution were: 5.3, 56, 172 ms and the fraction of events corresponding to each distribution 0.52, 0.25, and 0.23 respectively. (*C*) Open time distribution inside a burst in the presence of 3 nM IbTX from 8,395 events. Mean open time was 175 ms. (*D*) Close time distribution inside a burst in the presence of 3 nM IbTX. Mean closed times were 4.7, 60, and 235 ms with fraction of events 0.65, 0.31, and 0.04, respectively. Total number of events 8,394. In all cases the solid lines are the fitted probability density functions. Applied voltage 0 mV. Other experimental conditions as in Fig. 2.

constants for the bimolecular reaction described in scheme R1, under the experimental conditions of Fig. 5, are $k_1 = 4.4 \times 10^6\ M^{-1}\ s^{-1}$ and $k_{-1} = 4.9 \times 10^{-3}\ s^{-1}$ yielding and apparent dissociation constant, $K_d = 1.07$ nM. The next question we asked was whether the toxin was acting by occlusion of the external mouth of the channel.

## External tetraethylammonium (TEA) relieves IbTX blockade

The K(Ca) channel is blocked from the external side by submillimolar concentrations of the quaternary ammonium ion TEA (Latorre et al., 1982; Vergara et al., 1984; Yellen, 1984; Blatz and Magleby, 1984; Villarroel, 1989). TEA blocks the K(Ca) channel by binding to a receptor located in the extracellular channel mouth and thus hinders the flow of $K^+$ (Villarroel et al., 1989). Fig. 6 shows that addition of TEA to the external side besides causing a reduction in the channel current, it also produces a lengthening of the burst periods. Addition of TEA to a final concentration of 1 mM to the external side of the channel increased the $\tau_A$ from 113 s in the absence of TEA (3.6 nM IbTX) to 430 s. On the other hand, $\tau_B$ was 335 s in the absence of TEA and 386 s in the presence of the quaternary ammonium ion. If the channel (Ch) is not able to accommodate both IbTX and TEA simultaneously (i.e., the reactions are competitive), the IbTX block kinetic should follow the kinetic scheme:

$$\begin{array}{ccc} & + \text{IbTX} \rightleftarrows \text{ChIbTX} \\ \text{Ch} & & \\ + & & \\ \text{TEA} & & \\ \uparrow\downarrow & K_i & \\ \text{ChTEA}. & & \end{array} \qquad \text{(R2)}$$

The kinetic of TEA blockade is too fast compared with the time response of the current measuring circuit. Therefore, the block events are filtered and TEA appears to reduced the magnitude of the single-channel current. Be-

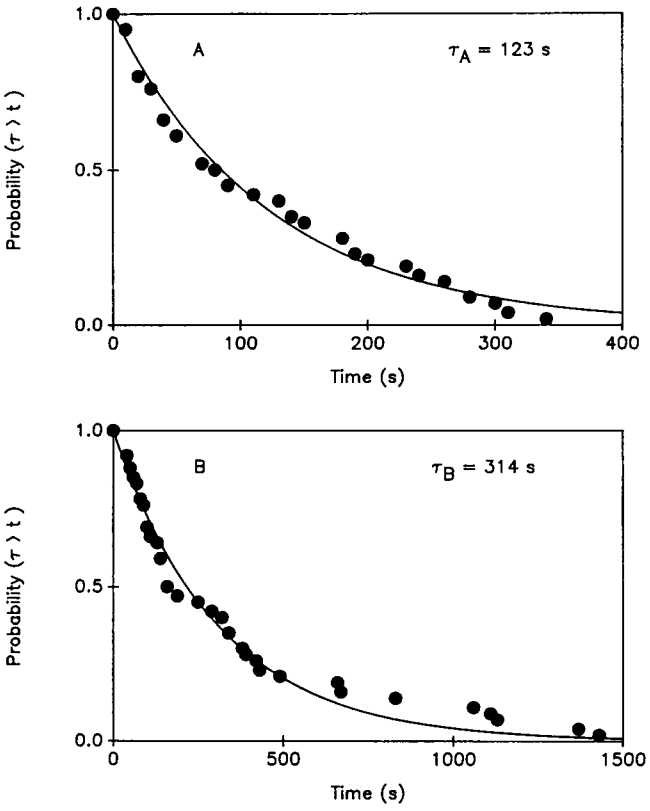


FIGURE 4 Cumulative dwell-time probability distributions for bursts and long-lived quiescent (blocked) times induced by IbTX. (*A*) Burst (active) time distribution. The solid line is the best fit to the data with an exponential distribution of dwell times. Mean active time, $\tau_A$, was 123 s. Regression coefficient: 0.9949. (*B*) Blocked time distribution. Best fit to the data was obtained with an exponential function with a mean blocked time, $\tau_B$, of 314 s. Regression coefficient: 0.9938. The internal ionic composition was 300 mM KCl, 5 mM MOPS-KOH, pH 7, and the external was 300 mM NaCl, 5 mM MOPS-KOH, 3.6 nM IbTX, pH 7. Holding potential was 0 mV. Number of events was 80.

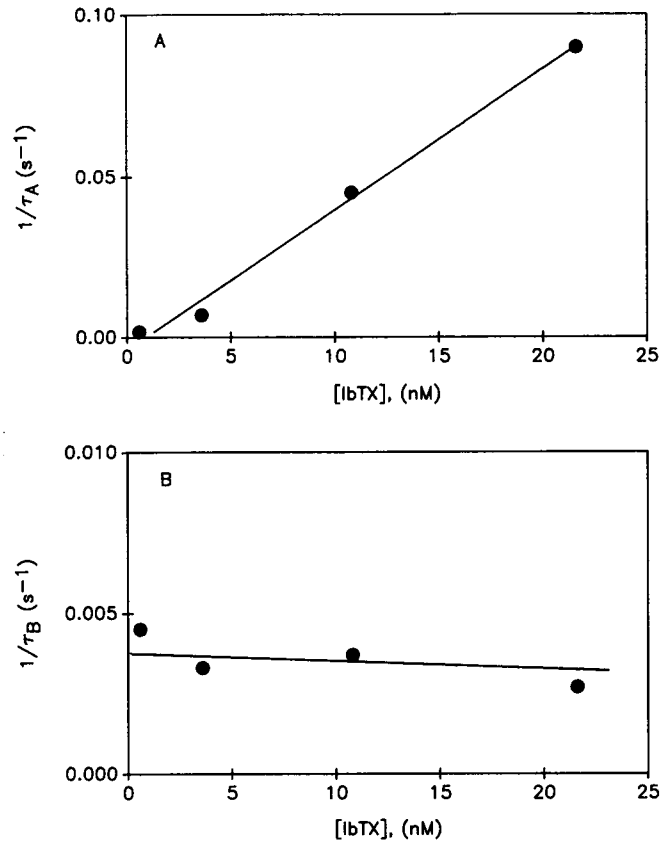


FIGURE 5 Blocking kinetics of IbTX. The reciprocal values of the mean active (*A*) and mean blocked (*B*) times were plotted as a function of IbTX concentration. Other experimental conditions as in Fig. 4.

cause of this effect, the ratio of the average single-channel current in the presence of TEA, $\langle i \rangle$, to the current measured in the absence of the fast blocker, $i_o$, is a direct measure of the probability, $P_{Ch}$, that the channel is not occupied by TEA and is given by the relation (Coronado and Miller, 1979; Villarroel et al., 1988; Latorre and Miller, 1983):

$$\langle i \rangle / i_o = P_{Ch} = \{1 + [TEA]/K_i\}^{-1}, \quad (3)$$

where $K_i$ is the dissociation constant for the TEA blocking reaction. On the other hand, according to scheme R2 and inasmuch as the TEA blocking reaction is in equilibrium with respect to the IbTX blocking reaction, $\tau_A$ in the presence of TEA is given by (Latorre and Vergara, 1983; Miller, 1988):

$$\tau_A(TEA) = 1/k_1[IbTX]P_{Ch}. \quad (4)$$

The expression for $\tau_B$, on the other hand, is as in Eq. 2. Combination of Eqs. 1, 3, and 4 gives:

$$\tau_A(TEA)/\tau_A = 1 + [TEA]/K_i = i_o/\langle i \rangle. \quad (5)$$

The measured values of $\tau_A(TEA)/\tau_A$ and $i_o/\langle i \rangle$ are 3.8 and 3.4, respectively, suggestive of a simple competitive relationship (i.e., Eq. 5) between IbTX and TEA.

## External $K^+$ modifies the IbTX association rate constant

Table 1 shows that the second order association rate constant is decreased as the external $[K^+]$ is increased. Changing the external $[K^+]$ from 12.5 to 310 mM promoted a 10-fold decrease in $k_1$ whereas the dissociation rate constant remained essentially constant. We think that this is an ionic strength effect rather than a specific effect of $K^+$ because the values for $k_1$ obtained in similar concentrations of $Na^+$ or $K^+$ are alike. A similar effect has been found for the binding of ChTX to K(Ca) chan-

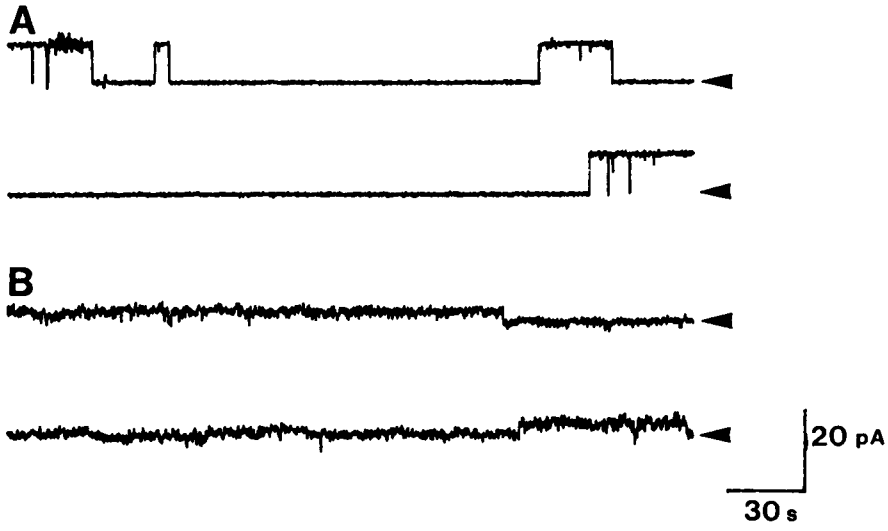


FIGURE 6 Effect of external TEA on IbTX block. A single K(Ca) channel was exposed to 3.6 nM external IbTX (*A*). (*B*) Same single K(Ca) channel, but after the addition of TEA to a final concentration of 1 mM to the external side. Other experimental conditions as in Fig. 3. $P_o$ in the control record and during bursts was 0.99.

nels (Anderson et al., 1988; Miller, 1990). Anderson et al. (1988) and Miller (1990) argued that a decrease in $k_1$ as the external ionic strength is raised is perfectly understandable in terms of a local electrostatic field induced by negative fixed charges located in the external channel entrance. The existence of such charges has been previously demonstrated (MacKinnon et al., 1989). Although, qualitatively the ionic strength effect is the same for both, ChTX and IbTX quantitatively there are dramatic differences; an increase from 20 to 150 mM promoted a 40-fold decrease in $k_1$ for the ChTX blocking reaction, a result that indicates that ChTX binding to the channel is more sensitive to ionic strength variations than that of IbTX.

## DISCUSSION

### Charybdotoxin and iberiotoxin mode of action

Charybdotoxin was the first high-affinity inhibitor directed against the large conductance $Ca^{2+}$-activated $K^+$ channel found (Miller et al., 1985; for recent reviews see Moczydlowski et al., 1988; Strong, 1990; Garcia et al., 1991). Charybdotoxin resulted to be a highly basic 37 amino acid residues peptide with its $NH_2$-terminal amino group blocked in the form of pyroglutamate (Gimenez-Gallego et al., 1988; Luchessi et al., 1989; Strong et al., 1989; see Fig. 1). ChTX with a $K_d$ of ~10 nM at physiological ionic strength, blocks K(Ca) channels following a simple bimolecular reaction in which blocking events are observed as a long-lived closed state (~20 s) (Miller et al., 1985; Anderson et al., 1988). Depolarizing voltages increase the rate of dissociation. However, the apparent voltage dependence of the blockade is lost when internal $K^+$ is replaced by an impermeant cation such as $Na^+$ (Anderson et al., 1988; MacKinnon and Miller, 1988). Taking together these results indicate that $K^+$ is able to enter a blocked pore from the internal side and destabilize the ChTX-channel complex. They also suggest that ChTX is actually plugging the channel by binding to a site located in the external mouth of it. This conclusion is supported by the fact that TEA inhibits the toxin effect in a strictly competitive manner (Miller, 1988; Garcia et al., 1991).

Our results provide strong evidence that IbTX inhibits K(Ca) channels by a mechanism very similar to that of ChTX. First, IbTX binds to the channel through a bimolecular reaction. Second, TEA alleviates the blockade induced by the toxin. However, the $K_d$ for IbTX under similar experimental conditions is about 10-fold smaller than that found for ChTX (~10 nM; Anderson et al., 1988; Miller, 1990). The difference between ChTX and IbTX regarding the external ionic strength sensitivity of the blocking reaction can be also explained in terms of toxin-channel electrostatic interactions. The negative charges located in the external mouth have the effect of increasing the toxin local concentration. This concentration is a function of the local field and of the net charge of the blocking toxin, because ChTX has a much higher net positive charge than IbTX it is expected than its local concentration should be a much steeper function of ionic strength (e.g., Aveyard and Haydon, 1973). The binding of ChTX is strongly electrostatic, the association rate constant for this toxin decreases 1,000-fold when the ionic strength is raised from 20 to 800 mM (Miller, 1990). According to Miller (1990) the local electrostatic effects induced by the presence of negatively charged groups in the channel mouth (MacKinnon and Miller, 1989; MacKinnon et al., 1989) are abolished if the ionic strength is raised above 500 mM. The values for $k_1$ (ChTX) obtained by Miller (1990) at 150 and 800 mM $K^+$ are $5 \times 10^6$ and $2 \times 10^5$ $M^{-1}$ $s^{-1}$. On the other hand, the value of $k_1$ for IbTX obtained at the highest ionic strength used in the present work (450 mM) is $1.7 \times 10^6$ $M^{-1}$ $s^{-1}$. Therefore, in the absence of unspecific electrostatic effects it appears that there is not a great difference in the rapidity at which both toxin reach their receptors producing a channel-toxin bound state. The main difference between IbTX and ChTX resides in the time the toxin dwells in the channel mouth. This is ~10 s for ChTX and 200 s for IbTX. i.e., IbTX binds to the K(Ca) from rat muscle channel tighter than ChTX. What are the structural differences that made IbTX to glue better to the K(Ca) channel than ChTX? As mentioned above, IbTX has a primary amino acid sequence having 70% homology with ChTX (Galvez et al., 1990; Garcia et al., 1991). The main difference resides in the amount of negatively charged residues; IbTX possess four aspartate residues in excess over ChTX (Fig. 1) making of this toxin a much less basic peptide. One simple explanation of the large difference in $k_{-1}$ between these two highly homologous toxins is that peptide charge (or their distribution) is crucial in determining the dissociation rate constant. In fact, MacKinnon and Miller (1988) found that in the absence of internal $K^+$ and at 37 mV, $k_{-1}$ for ChTX is predicted to be as low as 0.01 $s^{-1}$. This economical explanation is not supported by the experimental data obtained by Giangiacomo et al. (1991) because they found a 10-fold increase in $k_{-1}$ in going from 25 to 300 mM internal $K^+$. Thus, the difference in the dissociation rate constant between the two toxins must reside in other structural differences such as, for example, different foldings of the toxins promoted by the non-identical regions (Fig. 1).

Despite the similarities in their mode of action in bilayers, Galvez et al. (1990) found that ChTX binding to aortic sarcolemmal vesicles was inhibited by IbTX in a noncompetitive manner. This observation is puzzling because very similar results to those reported in the present study have been obtained in planar bilayers using a K(Ca) channel from aortic smooth muscle. It is possible that IbTX binds allosterically to a site present in the smooth muscle vesicles that is lost or modified during

vesicle fusion and the subsequent channel incorporation to the planar bilayer and/or that the iodinated ChTX used in the binding studies behaves altogether different than native ChTX.

## Toxin specificity

Evidence is mounting that ChTX is not specific for the large conductance $Ca^{2+}$-activated $K^+$ channel. For example, Farley and Rudy, 1988 and Reinhardt et al., 1989 found that ChTX is able to block both high and intermediate conductance $Ca^{2+}$-activated $K^+$ channels and the data of Hermann and Erxleben (1987) showed that ChTX also blocks a $Ca^{2+}$-activated $K^+$ channel of small conductance. Moreover, Reinhardt et al., (1989) found that a large conductance $Ca^{2+}$-activated $K^+$ channel albeit with a slow gating, is not blocked by ChTX. ChTX also inhibits a voltage-dependent $K^+$ channel from developing T cells in mouse thymocytes and T lymphocytes at very low concentrations (Lewis and Cahalan, 1988; Sands et al., 1989). Iberiotoxin, on the other hand, shows a high selectivity for K(Ca) channels. In $GH_3$ cells, IbTX does not alter A-type $K^+$, $Ca^{2+}$, or $Na^+$ currents, but completely inhibits the $Ca^{2+}$-activated $K^+$ currents present in this type of cells. Moreover, lymphocyte voltage-dependent $K^+$ channel are unaffected by IbTX (Garcia et al., 1991). IbTX, given its high specificity may proved to be an important tool to obtain information about the structure and function of the large conductance $Ca^{2+}$-activated $K^+$ channel.

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